

S. C. Lai · S. T. Jiang · K. M. Chen · H. H. Lee

## Matrix metalloproteinases activity demonstrated in the infective stage of the nematodes, *Angiostrongylus cantonensis*

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**Abstract** Ingestion of the larval nematode *Angiostrongylus cantonensis* can cause the human eosinophilic meningitis known as angiostrongyliasis. Analysis of the extracts and excretory-secretory (ES) products of *A. cantonensis* larvae and adult stages on gelatin substrate zymography demonstrated the presence of distinct gelatinolytic enzymes. In worm extracts, inhibitor studies showed that the metalloproteinases revealed in L<sub>1</sub> (23 kDa), L<sub>3</sub> (66, 42 and 30 kDa), young adult worm (72 and 94 kDa) and adult worm (72 and 94 kDa). In ES products, the L<sub>1</sub> revealed one low (42 kDa) and two high (105 and 94 kDa) molecular weight proteolytic bands that degraded gelatin in substrate gels. The L<sub>3</sub> revealed three low (66, 50, and 30 kDa) and one high (105 kDa) molecular weight proteolytic bands. Inhibitor studies confirmed that the 105 and 94 proteolytic bands of the L<sub>1</sub>, and the 50 and 30 kDa proteolytic bands of the L<sub>3</sub> classification were metalloproteinases. These metalloproteinases secreted in the infective larvae may be associated with the parasite dissemination or pathogenesis.

### Introduction

The parasitic nematode *Angiostrongylus cantonensis*, which dwells in the rat pulmonary artery, is the most common infectious cause of eosinophilic meningitis worldwide (Kliks and Palumbo 1992). The adult worm of males typically measure 14–15 mm in length, and has

a tail with copulatory bursa and long spicules. Females typically measure 24–26 mm in length, and present a characteristic barber-pole appearance (Lindo et al. 2002). The nematode has a complex life cycle (Alicata 1965). Eggs hatch in the lungs, and first-stage larvae (L<sub>1</sub>) pass out in the faeces, to subsequently enter a molluscan intermediate host where they moult twice. The third stage larvae (L<sub>3</sub>) of *A. cantonensis* typically measure 425–524 µm in length and 23–34 µm in width. The posterior end of the tail always terminates in a fine point (Ash 1970). The infective L<sub>3</sub> orally infect the final host and are carried in the blood to the central nervous system, where they moult twice to become immature adults. Once reaching the branches of the pulmonary artery, they grow rapidly, attain sexual maturity, and release eggs (Alicata 1965).

The production of proteolytic enzymes and their release as excretory-secretory (ES) products have been reported for various helminthes (Matthews 1982; McKerrow and Doenhoff 1988; McKerrow et al. 1990). Cysteine proteases have been found in *Ancylostoma caninum* (Harrop et al. 1995) and *Toxocara canis* (Loukas et al. 1998). Serine proteinases have been identified in *Anisakis simplex* (Morris and Sakanari 1994). Metalloproteinases have also been identified in a variety of helminthes including *Brugia malayi* (Petralanda et al. 1986), *T. canis* (Robertson et al. 1989), *Strongyloides stercoralis* (McKerrow et al. 1990), *Nippostrongylus brasiliensis* (Healer et al. 1991), *Dirofilaria immitis* (Richer et al. 1992), *Trichuris suis* (Hill et al. 1993), *A. caninum* (Hawdon et al. 1995), *Caenorhabditis elegans* (Wada et al. 1998) and *Gnathostoma spinigerum* (Uparanukraw et al. 2001).

Matrix metalloproteinases (MMPs) are a family of at least 20 zinc metallo-endopeptidases in vertebrates that regulate cell-matrix composition. They have been divided into subgroups according to their structure and function (Matrisian 1992). These enzymes are thought to participate in extracellular matrix (ECM) remodeling and degradation, and have been implicated in important roles during organ morphogenesis, embryonic

S. C. Lai · S. T. Jiang · K. M. Chen · H. H. Lee (✉)  
Department of Parasitology, Chung Shan Medical University,  
110, Section 1, Chien-Kuo North Road,  
402 Taichung, Taiwan  
E-mail: shih@csmu.edu.tw  
Tel.: +886-4-24730022  
Fax: +886-4-23823381

development and pathological processes (Stetler-Stevenson et al. 1993; Sato and Seiki 1996).

We have previously shown that the *A. cantonensis* could induce host MMP-9 production in the brain (Lai et al. 2004; Lee et al. 2004). However, knowledge about the MMPs in nematodes is limited and whether the proteinases can produce from *A. cantonensis* remains unclear. The present study sought to investigate the activity of metalloproteinases in the extracts and ES products of *A. cantonensis*.

## Materials and methods

### Parasite preparation

The L<sub>3</sub> (infective larvae for mammalian host) of *A. cantonensis* originally obtained from the field mollusk host that were propagated for several months in our laboratory by cycling through rats and snails (*Biomphalaria glabrata*). The larvae within tissues were recovered using a modification of the method of Parsons and Grieve (1990). Briefly, the shells were crushed, the tissues were homogenized, and digested in a pepsin-HCl solution (pH 1–2, 500 I.U. pepsin/g tissue), and incubated with agitation in a 37°C water bath for 2 h. Host cellular debris was removed from the digest by centrifugation at 1,400g for 10 min. The larvae in the sediment were collected by serial washing in double-distilled water and counted under the microscope. Immature adult worms were dissected from rat brains on day 25 post-inoculation (PI). Each brain was torn into small pieces and homogenized separately in 15 ml of 0.25% sodium citrate in phosphate-buffered saline (PBS) followed by centrifugation. Adult worms were obtained as described previously (Joshua et al. 1995). L<sub>1</sub> (infective larvae for mollusk host) were obtained by pepsin:HCl digestion of lungs obtained at 40 days PI, followed by purification from lung tissue by pelleting through 100% isopaque (Pharmacia, New York, NY) (Bessarab and Joshua 1997).

### Parasite extracts

Worms were thoroughly washed in PBS to free them of enzyme, ground in liquid nitrogen, homogenized in PBS in an ice bath and subjected to repeated brief sonications. The success of this procedure in completely breaking worms apart was confirmed microscopically. The extracts were centrifuged at 12,000g for 10 min.

### In vitro cultivation of *A. cantonensis*

For the experiments, five groups were used. All experiments utilized RPMI-1640 medium (Sigma, USA). The groups were: control (no worm); L<sub>1</sub>, 2000 L<sub>1</sub> cultured in medium; L<sub>3</sub>, 2000 L<sub>3</sub> cultured in medium; young adult, 60 young adult worms cultured in medium; adult, 60

adult worms (30 males and 30 females) cultured in medium. The control medium or worm-containing media were incubated at 37°C for 72 h in 200 µl of sterile medium, pH 7.2, containing 100 IU/ml penicillin and 100 µg/ml streptomycin. The motility of the worms was checked microscopically. The medium was changed every 24 h by pelleting the worms at 150g for 5 min and resuspending them in fresh complete medium. The culture medium was checked for bacterial contamination during each experiment. Bacterial contamination was prevented prior to the experiments by passing the medium through a 0.2 µm filter (Millipore, Eschborn, Germany) and storing the filtrate at –70°C before use.

### Experimental animals and infection

Five-week-old male rodents, BALB/c mice and Sprague-Dawley rats, were purchased from the National Laboratory Animal Center, Taipei, Taiwan. They were maintained at 12 h light/dark cycle photoperiods, provided with Purina Laboratory Chow and water ad libitum, and kept in our laboratory for more than one week before the experimental infection. The rodents were prohibited food and water for 12 h before infection. They were infected with 60 *A. cantonensis* L<sub>3</sub> by oral inoculation and sacrificed on day 25 post-infection (PI). Control rodents received only water and were also sacrificed on day 25 PI.

### Gelatin substrate zymography

The *A. cantonensis* samples (extracts and cultured fluids, 20 µg protein) were diluted 1:1 in sample buffer (1% SDS, 2% glycerol, 10% bromophenol blue and 0.5 M Tris-HCl, pH 6.8). Samples were loaded on 7.5% (mass/volume) SDS-polyacrylamide gels that had been copolymerized with 0.1% gelatin (Sigma, USA). Stacking gels were 4% (mass/volume) polyacrylamide and did not contain gelatin substrate. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature at 120 V for 1 h. The gel was washed two times at room temperature for 30 min each in 2.5% Triton X-100, and then washed two times with double-distilled water for 10 min each. The gel was incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, 10 mM CaCl<sub>2</sub>, 0.02% Brij-35, 0.01% NaN<sub>3</sub>) at 37°C for 18 h. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma, USA) for 1 h and destained in 15% methanol/7.5% acetic acid. Gelatinase activity was detected as unstained bands on a blue background.

### Inhibition of gelatinase on gelatin zymography

Samples were resolved by SDS/PAGE on 7.5% polyacrylamide gels impregnated with 0.1% gelatin

(Sigma, USA). Following electrophoresis, gels were soaked in 2.5% Triton-X-100 to replace SDS, washed twice with water, then incubated at 37°C for 18 h in MMP activation buffer (50 mM Tris, pH 8.0, 5 mM CaCl<sub>2</sub>). In control experiments, calcium was replaced with 10 mM ethylenediamine tetraacetic acid (EDTA, Sigma, USA) in the activation buffer. For inhibitor studies, either 20 μM leupeptin (Sigma, USA), 2 mM phenylmethanesulphonyl fluoride (PMSF, Sigma, USA), 5 mM 1,10-phenanthroline (Sigma, USA), solvent was added to the Triton and activation buffers. Zymography gels were stained with Coomassie Brilliant Blue and destained in 15% methanol/7.5% acetic acid. Proteins with gelatinolytic activity were revealed as clear bands on a blue background.

## Results

### Gelatinase activity from worm extracts

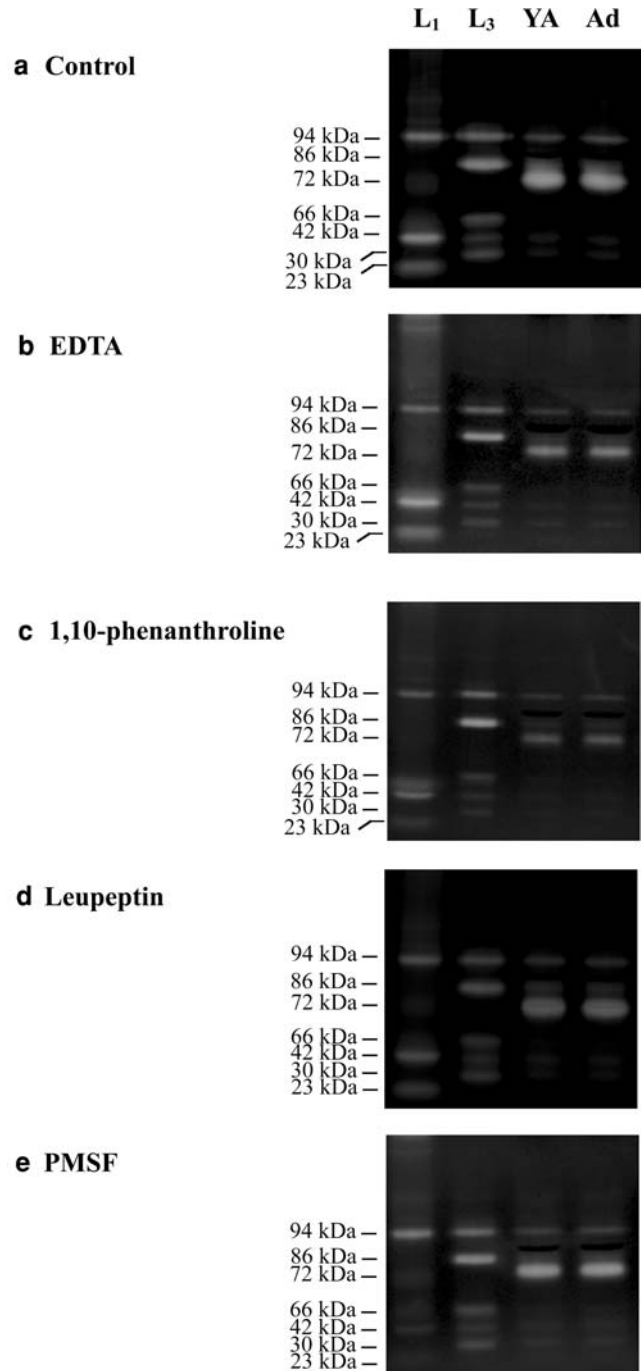
*Angiostrongylus cantonensis* L<sub>1</sub> extracts had a restricted profile when examined by gelatin substrate SDS-PAGE. Three dominant bands at 94, 42 and 23 kDa. In L<sub>3</sub> extracts, five gelatinase bands were evident at 94, 86, 66, 42 and 30 kDa. In extracts from both young and adult worms two gelatinase bands were observed at 94 and 72 kDa (Fig. 1a). Inhibitor studies showed the 23 kDa L<sub>1</sub> gelatinase band share characteristics of metalloproteinase/cysteine-proteinase/serine-proteinase, and the 42 kDa band was serine-proteinase. In L<sub>3</sub> gelatinase bands, the 66, 42 and 30 kDa bands showed share characteristics of metalloproteinase/cysteine-proteinase. The 72 and 94 kDa gelatinase bands in the young adult worm and adult worm showed share characteristics of metalloproteinase/cysteine-proteinase (Fig. 1b–e).

### Gelatinase activity from worm ES products

Analysis of L<sub>1</sub> ES products revealed one low (42 kDa) and two high (105 and 94 kDa) molecular weight proteolytic bands that degraded gelatin in substrate gels. Similar analysis of L<sub>3</sub> products revealed three low (66, 50, and 30 kDa) and one high (105 kDa) molecular weight proteolytic bands. In contrast, gelatinase activities were not detected in the ES obtained from young adult and adult worms (Fig. 2a). Inhibitor studies showed the 105 and 94 kDa L<sub>1</sub> gelatinase bands, and 50 and 30 kDa L<sub>3</sub> gelatinase bands were metalloproteinases (Fig. 2b–e).

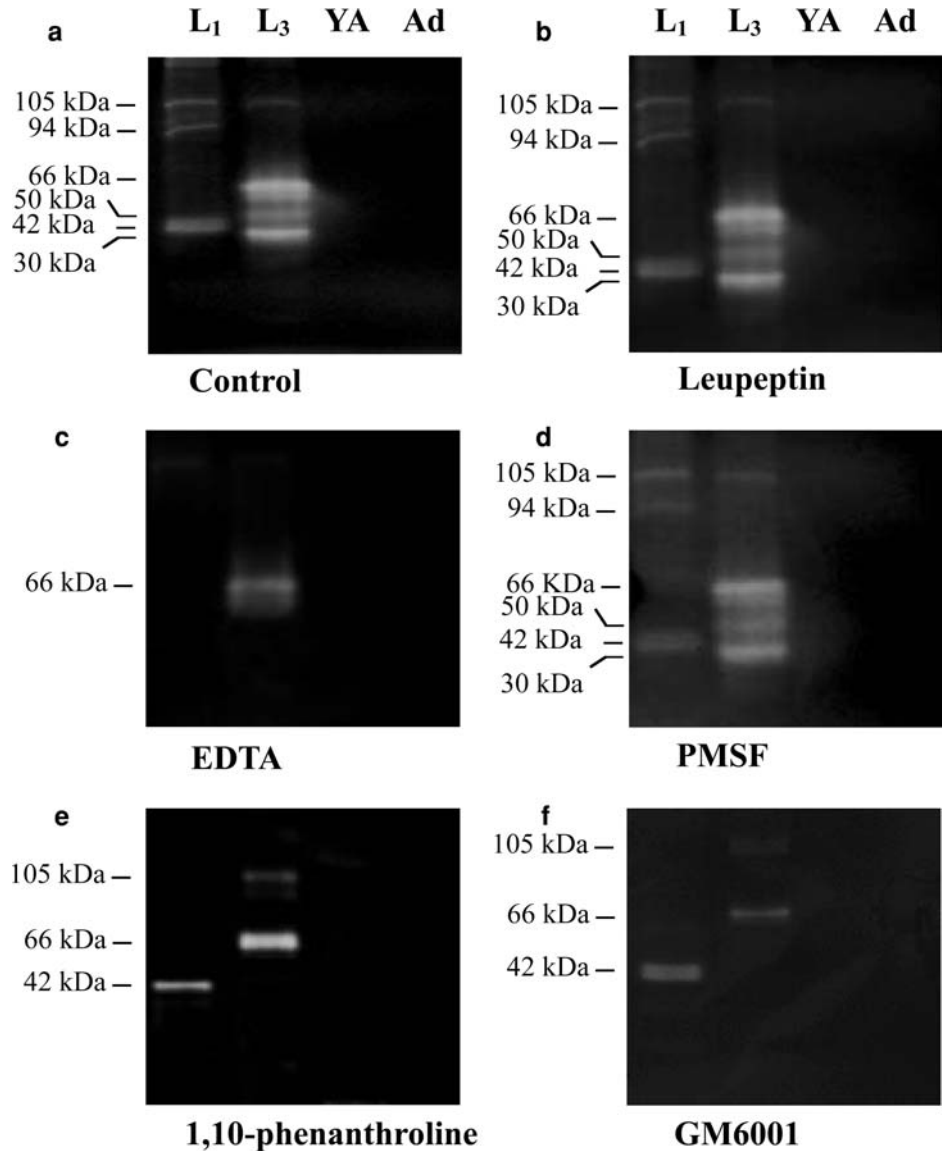
### Differentiation the gelatinases from worms and hosts

The gelatinase bands of 94 (MMP-9) and 72 (MMP-2) kDa were detected in 60 *A. cantonensis*-infected mice but not in rats or following culturing of 60 worms. Cere-



**Fig. 1** Gelatinase activity from worm extracts. **a** L<sub>1</sub> of *Angiostrongylus cantonensis* extracts have a restricted profile when examined by gelatin substrate SDS-PAGE, with three dominant bands at 94, 42 and 23 kDa; five gelatinase bands at 94, 86, 66, 42 and 30 kDa of the L<sub>3</sub>; two gelatinase bands at 94 and 72 kDa in young adult and adult worms. **b–e** In L<sub>1</sub> gelatinase bands, the 23 kDa band was significantly inhibited by 1,10-phenanthroline, leupeptin or PMSF; the 42 kDa band was significantly inhibited by PMSF. In L<sub>3</sub> gelatinase bands, the 66, 42 and 30 kDa bands were significantly inhibited by 1,10-phenanthroline or leupeptin. The 72 kDa gelatinase bands in the young adult worm and adult worm were significantly inhibited by EDTA and 1,10-phenanthroline, partially inhibited by leupeptin. The 94 kDa gelatinase bands in the young adult worm and adult worm were significantly inhibited by leupeptin, partially inhibited by EDTA or 1,10-phenanthroline. Other gelatinase bands from worm extracts could not be inhibited by EDTA, 1,10-phenanthroline, leupeptin or PMSF

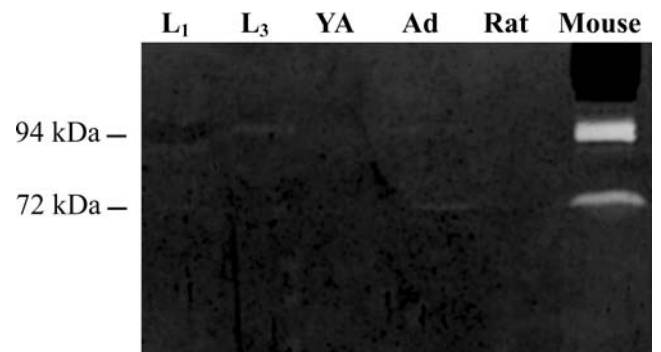
**Fig. 2** Gelatinase activity from worm secretory products. **a** The analysis of the excretory-secretory products of the L<sub>1</sub> revealed one low (42 kDa) and two high (105 and 94 kDa) molecular weight proteolytic bands that degraded gelatin in substrate gels. In addition, the L<sub>3</sub> revealed three low (66, 50, and 30 kDa) and one high (105 kDa) molecular weight proteolytic bands. The 105 and 94 kDa gelatinase bands of the L<sub>1</sub>, and the 50 and 30 kDa gelatinase bands of the L<sub>3</sub> were inhibited by EDTA (**b**), 1,10-phenanthroline (**c**), and GM6001 (**f**), but not affected by leupeptin (**d**) or PMSF (**e**)



brospinal fluid from mice infected with *A. cantonensis* contains metalloproteinase. However, MMPs were not detected in experiments utilizing 60 larvae, 60 young adult worms, or 60 adult worms (Fig. 3).

## Discussion

Proteinases serve a variety of functions in parasite development including the facilitation of host tissue invasion, digestion of host proteins, inhibition of blood clotting, molting and evasion of the host immune response (McKerrow 1989). In the present study, we observed metalloproteinases secretion in the L<sub>3</sub> (50 and 30 kDa). These molecular weights and responses to the inhibitory agents are consistent with the classification of these species as metalloproteinases. These bands were observed in gelatin zymography after proteolysis by



**Fig. 3** Differentiation of gelatinase from worms and hosts. The gelatinase bands of 94 and 72 kDa were detected in *Angiostrongylus cantonensis*-infected mice. Gelatinase activity could not be detected in rats or in the 60 worm homogenates of L<sub>1</sub>, L<sub>3</sub>, young adult and adult worms, respectively

ES products from *A. cantonensis*. This means that metalloproteinases secreted by *A. cantonensis* L<sub>3</sub> exhibit diversity based on the differential migration in polyacrylamide gels. These enzymes may play a role in the parasite penetration of the stomach or intestinal wall of the host, although more directed investigations will be needed to confirm these roles.

Cerebrospinal fluid from mice infected with *A. cantonensis* also contains metalloproteinase. Earlier studies in our laboratory demonstrated that, following infection with 60 *A. cantonensis*, MMP-9 can be induced in ICR mice (Lai et al. 2004) and BALB/c mice (Lee et al. 2004). Presently, at least 2000 larvae (L<sub>1</sub> or L<sub>3</sub>) were required for the detection of the enzyme activity. In contrast, MMPs were not detected in 60 *A. cantonensis* of L<sub>1</sub>, L<sub>3</sub>, young adult worms or adult worms, respectively. These data can differentiate the metalloproteinases in this study secreted by the L<sub>3</sub>, young adult or adult worms, and previous studies (Lai et al. 2004; Lee et al. 2004) secreted by the mice. Moreover, our findings strongly support the suggestions that the production of metalloproteinases is by the host rather than the parasite (Hotez et al. 1985; Petralanda et al. 1986; Knox and Kennedy 1988; Lackey et al. 1989).

Proteinase secreted by the infective larvae facilitates penetration of skin or intestinal walls of humans (Tort et al. 1999). Metalloproteinase-mediated degradation of ECM components is also a feature of some helminthes (Petralanda et al. 1986). Ingestion of larval nematode *A. cantonensis* can cause the human disease known as angiostrongyliasis (Alicata 1965). After ingestion, *A. cantonensis* larvae can be invasive, penetrating host stomach or intestinal wall (Wang et al. 1991). Thus, we suggest that metalloproteinases secreted in the infective larvae may be associated with the parasite dissemination or pathogenesis.

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